An aging-susceptible circadian rhythm controls cutaneous antiviral immunity

Stephen Kirchner, …, Amanda S. MacLeod, Jennifer Y. Zhang

*JCI Insight. 2023. [https://doi.org/10.1172/jci.insight.171548](https://doi.org/10.1172/jci.insight.171548)*

**Graphical abstract**

Find the latest version:

[https://jci.me/171548/pdf](https://jci.me/171548/pdf)
An Aging-Susceptible Circadian Rhythm Controls Cutaneous Antiviral Immunity

Stephen Kirchner¹,², Vivian Lei¹, Paul T. Kim¹, Meera Patel¹, Jessica L. Shannon¹,³, David Corcoran⁴, Dalton Hughes⁵, Diana K. Waters⁵, Kafui Dzirasa⁵,⁶,⁷,⁸,⁹, Detlev Erdmann¹⁰, Jörn Coers²,³, Amanda S. MacLeod¹,²,³,¹¹, Jennifer Y. Zhang¹,¹² *

¹ Department of Dermatology, Duke University, Durham, NC, USA
² Department of Molecular Genetics and Microbiology, Duke University, Durham, NC, USA
³ Department of Immunology, Duke University, Durham, NC, USA
⁴ Duke Center for Genomic and Computational Biology, Duke University, Durham, NC, USA
⁵ Department of Neurobiology, Duke University, Durham, NC, USA
⁶ Department of Psychiatry and Behavioral Sciences, Duke University, Durham, NC, USA
⁷ Department of Biomedical Engineering, Duke University, Durham, NC, USA
⁸ Department of Neurosurgery, Duke University, Durham, NC, USA
⁹ Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, USA
¹⁰ Department of Surgery, Division of Plastic, Maxillofacial and Oral Surgery, Duke University, Durham, NC, USA
¹¹ Present address: Janssen Pharmaceuticals, San Diego, CA, USA
¹² Department of Pathology, Duke University, Durham, NC, USA.

*Corresponding author: Jennifer Y. Zhang, Duke Box 103052, Durham, NC 27710

Jennifer.zhang@duke.edu

Conflict of Interest:

A.S.M. consulted and received funds from Silab, but this funding was not directly used for this study. All other authors declare noncompeting interests.
ABSTRACT
Aged skin is prone to viral infections, but the mechanisms responsible for this immunosenescent immune risk are unclear. We observed that aged murine and human skin expressed reduced antiviral proteins (AVPs) and circadian regulators including Bmal1 and Clock. Bmal1 and Clock were found to control rhythmic AVP expression in skin and such circadian-control of AVPs was diminished by disruption of immune cell interleukin 27 signaling and deletion of Bmal1/Clock genes in mouse skins, as well as siRNA-mediated knockdown of CLOCK in human primary keratinocytes. We found that treatment of circadian enhancing agents, nobiletin and SR8278, reduced infection of herpes simplex virus 1 (HSV1) in epidermal explants and human keratinocytes in a BMAL1/CLOCK-dependent manner. Circadian enhancing treatment also reversed susceptibility of aging murine skin and human primary keratinocytes to viral infection. These findings reveal an evolutionarily conserved and age-sensitive circadian regulation of cutaneous antiviral immunity, underscoring circadian restoration as an antiviral strategy in aging populations.
INTRODUCTION

The skin acts as a physical barrier to invading pathogens, which can be disrupted by genetic defects, environmental challenges, wounds, and micro-injuries(1). Skin barrier disruptions are of special concern for elderly patients due to the reduced regenerative capacity in aged skin. Consequently, these patients experience an increased risk of pathogen infection and other clinical issues(2, 3). Nevertheless, how an aged skin microenvironment affects barrier function and immunosenescence is not well-understood. Aspects of the skin microenvironment that influence barrier defense include location of disruption, microbial content, moisture status, and age of the skin(3). In addition, the time at which a wound is inflicted changes barrier responses and results in differential healing rates(4), suggesting that the circadian rhythm in skin regulates tissue regeneration and immune responses.

The circadian rhythm controls time-of-day biological responses and regulates components of cell proliferation and wound re-epithelialization(5). Mice deficient in Bmal1, one of the core transcription factors of the circadian clock, exhibit greater burden in viral infections(6, 7), indicating that circadian rhythms influence antiviral functions. Circadian function declines in older individuals(8); however, aging circadian rhythms have not previously been characterized in the context of immunosenescent cutaneous barrier defenses.

Interleukin 27 (IL-27), a member of the IL-12 family of heterodimeric cytokines, was recently implicated in cutaneous defense against Zika virus(9). In response to skin injury, CD301b⁺ leukocytes are rapidly recruited to the wound site(10), and produce IL-27 which subsequently potentiates wound closure and induces production of innate antiviral proteins (AVPs)(9, 11). AVPs encompass several families, including oligoadenylate synthetase (OAS1, OAS2, OAS3), Myxovirus resistance proteins (MX1 and MX2), and interferon-induced transmembrane (IFITM)
family proteins(1). Circadian rhythms have been implicated in interferon-stimulated gene responses in several tissues, including skin and lung(12, 13). However, it is unclear if skin barrier antiviral function is influenced by circadian rhythms.

In this study, we discovered that circadian factors Bmal1 and Clock decrease in aged skin. We also found that circadian dysregulation impairs cutaneous AVP expression via epidermal keratinocyte-autonomous and leukocyte-derived cytokine-mediated processes. Our studies show that murine cutaneous AVPs are regulated by Bmal1 and Clock in intact and wound states. We found that circadian-mutant and aged mice exhibited reduced IL-27 expression in skin wounds. Additionally, IL-27, along with type I interferon signaling, is required for time-of-day dependent circadian-regulation of wound-induced AVP production. We demonstrate that genetic loss-of-function of circadian factors sensitized skin and keratinocytes to HSV1 infection, whereas agents such as SR8278 and nobiletin that increase circadian rhythm amplitudes in other tissues(14), enhanced cutaneous circadian rhythms and reduced HSV1 viral burden in human keratinocytes and epidermal explants. Finally, we demonstrate that these circadian agents have antiviral effects in aging murine skin and human skin cells.

RESULTS

Aging skin displays a decline in circadian rhythm and antiviral immune function.

The link between aging, cutaneous circadian rhythms, and barrier defense is unclear. To address this, we first investigated expression of circadian factors in murine skin of varying ages. We found that Bmal1, Clock, and Per2 were downregulated in aged (greater than 12 months) skin compared to young (approximately 3-6 months) skin (Figure 1A). Serial passaging of primary human keratinocytes, which acts as a surrogate for human skin aging(15), showed that circadian
transcriptional activity decreased with increasing passage numbers (Figure 1B). This is also corroborated in an existing human skin dataset, where ARNTL(BMAL1) appears to peak in middle age before declining in expression(16).

We next investigated whether aging skin exhibits deficiency in antiviral protein (AVP) production. Using quantitative RT-PCR and immunofluorescence, we found that mRNA and protein levels of AVPs (Oasl, Oas2, Ifitm1) were significantly reduced in aged murine skin compared to young skin (Figure 1C-D) and visualized in both epidermal structures and sebaceous glands(17). Barrier disruption triggers an AVP response in young skin(9, 11, 18). We observed that wounding significantly elevated AVP induction at 24-hours post wounding in young and old skin; however, the magnitude of induction is significantly higher in wounds of young skin compared to that of aged skin (Figure 1E).

AVPs are induced in the skin wound microenvironment by IL-27, a cytokine produced by CD301b+ leukocytes of the skin(9, 11). To address if aging impacts CD301b+ signaling, we examined murine skin across ages for the presence of CD301b+ cells via immunostaining and found that CD301b+ cells were reduced in the skin of aged compared to younger mice (Figure 1f). Flow cytometry analysis revealed that wounded aged skin had a decreased influx of CD301b+ cells and expression of IL-27 (Figure 1G-H) (Gating strategy shown in Supplementary Figure S1). IL-27 works in concert with type I interferon in inhibiting Zika virus infection(9). To test if type I interferon signaling is also affected by aging, we performed quantitative RT-PCR comparing the expression levels of type I interferons and their receptor, Ifnar1, in wounds of young and aged mice. We found no differences in Ifna2, Ifnβ, Ifna4, and Ifna11 between old and young skin wounds collected 24 hours post-wounding (Supplementary Figure S2). This data supported a link between aging, cutaneous rhythms, and antiviral barrier defense.
Cutaneous circadian rhythms regulate antiviral proteins.

Next, we asked if circadian decline could mechanistically be responsible for this immunosenescent barrier defense. To test the circadian-innate immunity link in the context of skin, we queried published microarray gene expression data of murine skin harvested every 4 hours (19). We scaled and clustered the expression of AVPs across Zeitgeber (standardized time-of-day) time-points within a 24-hour span using an additional dataset (19) and separated the genes into 5 clusters of distinct expression profiles. We found a variety of antiviral genes whose pattern of expression coincided with Bmal1(Arntl) in murine skin (Supplementary Figure S3). One such gene, Oas1a, was found to have a temporal expression pattern coincident with Arntl changes (Figure 2A). We then used a CIRCOS plot and determined a broader time-of-day regulation of antiviral immune genes in the skin (Supplementary Figure S3). This was further supported by findings in baboon skin (20) (Supplementary Figure S3), where a number of antiviral genes including IFIT2 had rhythmic expression similar to ARNTL (Figure 2B).

To validate this computational data and test if these AVP fluctuations are linked to circadian factors, we harvested belly skin from wild type and Bmal1−/− C57BL/6J mice at 8AM and 8PM time-points. We found that the basal expression of AVPs varied in the skin, as shown by qRT-PCR (Supplementary Figure S3). In agreement with this data, AVPs were reduced in Bmal1−/− skin compared to wild type skin as measured via qRT-PCR for Oas1a and Oas2 (Figure 2C) and immunostaining for Oas1a (Figure 2D). Similar results were obtained by qRT-PCR in the skin of circadian-deficient ClockΔ19 mutant mice (21) where circadian mutants expressed less AVP than their wild type littermates (Figure 2E).
To further examine the link between circadian genes and AVPs in the context of barrier disruption, we first compared AVP induction between wild type skin wounds collected at 8AM and 8PM, 24 hours post-wounding. *Oas1a* was significantly higher in 8PM wounds than that of 8AM wounds (Figure 2F); *Ifitm1* exhibited a similar trend, though did not reach significance. Compared to wild type counterparts, ClockΔ19 mutant skin exhibited a significant decrease of wound-induced AVP production across multiple time-points (Figure 2G). These data supported a link between circadian rhythms and antiviral proteins of the skin.

**Circadian regulation of AVP-induction requires IL-27 and type I interferon signaling.**

Cytokine production and leukocyte trafficking are well-characterized immune phenotypes with a circadian level control(22). We examined IL-27 expression in *Bmal1<sup>−/−</sup>* and *Bmal1<sup>+/−</sup>* mouse skin, aged around 1 month, in an existing dataset(19). We noted decreased expression of IL-27 along with other interferon-responsive antiviral genes such as *Oas1a, Ifitm1, Ifitm7*, and *Ifit3b* for intact *Bmal1<sup>−/−</sup>* skin (Figure 3A). Immunostaining revealed that numbers of CD301b<sup>+</sup> cells were reduced for intact *Bmal1<sup>−/−</sup>* skin compared to wild type counterparts (Figure 3B). Flow cytometry analysis confirmed the decrease of CD301b<sup>+</sup> cells in *Bmal1<sup>−/−</sup>* skin wounds and a reduced median fluorescence intensity (MFI) of IL-27p28 in these cells (Figure 3C-D, Supplementary Figure S4). To determine whether IL-27 is required for circadian-regulation of AVP induction, we used a Cre-loxP mouse model to ablate *Il27p28* in Lysozyme M-expressing (LysM-Cre) myeloid cells including CD301b<sup>+</sup> cells(11). We observed that deletion of IL-27 in myeloid cells markedly diminished the time-of-day response of wound-induction of AVPs (Figure 3E, Supplementary Figure S5).

To test if type I interferon has a circadian wound effect, we wounded *Ifnar1<sup>−/−</sup>* mice at 8AM and 8PM. We found that *Ifnar1* loss blunted significance of temporal variation in AVP expression...
(Figure 3F, Supplementary Figure S5). However, transcription of type I interferons, including Ifna2, Ifna4, Ifna11, and Ifnb, did not change with respect to time-of-day when measured 24 hours post-wounding (Figure 3G). These data supported a leukocyte mechanism of action for circadian-AVP function.

**Keratinocyte-autonomous circadian rhythm regulates antiviral protein transcription and cutaneous defense against herpes simplex virus 1 infection.**

Cell-autonomous immune defects are present in circadian-deficient fibroblast cultures with respect to viral infection(6, 7), but it is unclear if other skin cells contribute to this phenotype. We asked whether keratinocyte-autonomous circadian rhythms contribute to the observed AVP regulation. To address this question, we synchronized circadian clocks of primary human epidermal keratinocyte cultures via an overnight incubation with omission of growth factor supplements. As expected, clock synchronization induced an oscillatory pattern of BMAL1 expression (Figure 4A), coinciding with the oscillation of OAS1, OAS2, and MX1 antiviral genes that approximate a cosinor sine model (Supplementary Figure S6). To establish a direct link between circadian factors and AVP expression, we performed siRNA-mediated knockdown of BMAL1 (siBMAL1) and CLOCK (siCLOCK) in an immortalized NTER keratinocyte culture. By qRT-PCR, we found that gene silencing of BMAL1 and CLOCK significantly reduced expression of antiviral proteins (Figure 4B). Furthermore, this effect had direct effects on viral replication. When primary keratinocytes with siBMAL1 and siCLOCK were infected with herpes simplex virus type 1 (HSV1), they produced more virus than nonsilenced Control (siCtrl) keratinocytes as measured by PCR of viral gene UL29(Figure 4C). This was corroborated via immunofluorescence using NTERT keratinocytes, where circadian disruption was associated with significantly increased HSV1 antigen levels (Figure 4D-F).
**Circadian enhancement leads to decreased HSV1 infection in the skin.**

We hypothesized that enhancement of circadian function increases antiviral immunity of the skin. We expressed a Bmal1 promoter-driven luciferase reporter (23) in NTERT keratinocytes and validated that Bmal1-reporter expression is regulated in a circadian-dependent manner (**Figure 5A**). Using the Bmal1-reporter system, we found that treatment of 10 μM SR8278, a small molecule REV-ERB antagonist previously shown to increase circadian rhythms in non-skin tissues (24), enhanced the amplitude of rhythmic BMAL1 activity (**Figure 5A-B**).

To test if circadian augmentation has an antiviral effect, we utilized surgically discarded human skin samples. We separated human epidermis from dermis, infected the epidermis ex vivo with HSV1 in an air-liquid interface culture system and treated the epidermal explant culture with either vehicle control or 10 μM SR8278 for 24 hours. By immunofluorescent staining and quantification, we observed that SR8278 treatment significantly reduced HSV1 antigen expression in the epidermis (**Figure 5C-D**). We confirmed this reduction via qPCR for HSV1 UL29 gene with human K14 gene used for internal control (**Figure 5E**). We then asked whether the antiviral effect of SR8278 was dependent on circadian and antiviral factors. We performed siRNA-mediated gene silencing of BMAL1 in NTERT keratinocytes and visualized HSV1 using immunofluorescence after treatment with SR8278 (**Figure 5F**). After controlling for cell number by nuclei staining using Fiji (ImageJ), we found that SR8278’s antiviral effect was lessened in siBMAL1 transfected cells (**Figure 5G**). To confirm these findings, we infected NTERT keratinocytes with siRNA-mediated gene silencing of BMAL1 and CLOCK. qPCR analysis of the viral gene UL29 revealed that BMAL1 and CLOCK gene silencing significantly increased virus in culture media of SR8278 treated cells (**Figure 5H**), indicating that SR8278’s antiviral effect is predicated on circadian function. Interestingly, when we suppressed antiviral proteins OAS1 and
IFITM1 via siRNA in NTERT keratinocytes, SR8278’s effect was also lessened (Supplementary Figure S7), suggesting SR8278’s antiviral effect is both circadian and antiviral protein dependent.

We subsequently examined if other circadian augmenting compounds would have antiviral effects. Nobiletin is an antioxidant flavonoid with multiple pharmacological effects, including antioxidant properties(25), as well as an ROR agonist that potentiates circadian rhythms(14). Using the NTERT Bmal1-luciferase cell line, we confirmed that nobiletin increased Bmal1-reporter expression in keratinocytes (Supplementary Figure S7A). Treatment of nobiletin decreased HSV1 infection of human epidermal explants (Supplementary Figure S7B-D), suggesting an antiviral activity of circadian enhancers.

**Viral infections in aging skin are reduced by circadian augmentation.**

Aging skin is subject to immunosenescence and is known to be susceptible to viral infections. Thus, we examined if circadian modulation has an antiviral activity in aging skin. We found that 1 year-aged Bmal1+/− mutant aging skin, which displays premature aging, also show deficiency of antiviral protein transcription in the wound environment (Figure 6A). Following this pattern of antiviral deficiency, we infected epidermal explants of Bmal1+/− and WT animals with HSV1. By qPCR, we found that HSV1 levels were higher in Bmal1+/− epidermis than WT counterparts (Supplementary Figure 9). Next, we examined the effects of aging on viral infection. We infected aged and young WT murine epidermal explants with HSV1 and found via qPCR that HSV1 levels were higher in aged skin than that of younger skin (Figure 6B). Treatment of circadian drug SR8278 reduced HSV1 viral load in aged skin by roughly 50% as measured by qPCR (Figure 6C). Finally, we tested infection susceptibility of passaged human keratinocytes as a pseudoaging model. We found that human primary keratinocytes at P8 produce more virus than P2 keratinocytes (Figure 6D) and that this increased viral replication could be suppressed by
treatment of SR8278 (Figure 6E). These data indicate that circadian augmenting agents have antiviral effects on aging skin.

**DISCUSSION**

Our data reveal a pharmacologically tractable model of age-mediated circadian regulation of antiviral immunity of the skin. Skin aging leads to decline of circadian function in the skin, compromising epidermal and dermal antiviral responses. Pharmacological agents that potentiate skin cell circadian amplitude improve immunity via antiviral protein effect. Our data delineate new mechanisms responsible for the immunosenescent decline of antiviral immunity in aging skin, underscoring the circadian pathway as a new therapeutic target for enhancing aging skin barrier function.

Previous studies have shown that interferon stimulated genes are affected by skin circadian rhythms through a TLR7-dependent stimulation(12). We have demonstrated that type I interferon signaling is required for activation of antiviral immune responses but may not convey time-of-day responses. However, type I interferon signaling is required for maximum AVP expression irrespective of time-of-day, suggesting that the interferon pathway as the primary regulator of AVPs is subject to regulation by circadian factors. In this regard, STAT1 and STAT3, transcription factors involved in interferon response, display time-of-day-responses(26), and could be an indirect regulatory step between circadian transcription factors and antiviral proteins. Circadian factors may directly regulate AVPs by binding to the E-box consensus elements which are present in gene promoters of antiviral genes, such as *Oas1*, *Oas2*, and *Ifitm1*. Such possibilities may be explored via skin cell-specific ChIP-Seq for BMAL1 and CLOCK(27). These experiments may also help
explain why certain AVPs in primate skin follow distinct temporal expression as AVPs may experience differential BMAL1/CLOCK binding efficacies in the skin.

Our data show that CD301b⁺ leukocyte-derived IL-27 is important for the time-of-day-dependent response of AVP expression. It will be important to determine whether the decreased dermal infiltration of CD301b⁺ leukocytes in aged skin and Bmal1⁻/⁻ skin is a result of central or local circadian decline, and if rescuing this defect can restore circadian AVP functionality. Knowing specifically how the circadian rhythm acts on circulating immune cells and skin resident cells will provide better insights into how to leverage circadian rhythms for improving cutaneous tissue regeneration and defense against infection in aging skin. This information may also prove useful in understanding skin infections that resist traditional interferon induced immunity such as monkey pox(28).

We focused on a common skin pathogen HSV1, but intriguingly, different viruses may interplay with circadian rhythms in distinct fashions. For example, respiratory syncytial and vesicular stomatitis virus replication rates were found to have opposite responses to circadian deletion(7), possibly due to how these viruses differ in viral entry and replication machinery. Other work has shown that time of day impacts response to HSV-2 in murine skin, as well as treatment responses (29). Our data show that siRNA-mediated gene silencing of BMAL1/CLOCK sensitized human keratinocytes to HSV1 infection and diminished protective effects conferred by pharmacological circadian enhancers (SR8728 and nobiletin). In agreement with our data, Bmal1⁻/⁻ mice exhibit greater HSV1 viral replication than wild type counterparts(6), which is in part attributed to aspects of host cell-virus interaction, such as intracellular trafficking and chromatin assembly. Paradoxically, BMAL1 and CLOCK are found to be hijacked by viral proteins to support viral replication(30). How to best balance the antiviral and pro-viral activity of circadian
factors requires further study and should incorporate more skin-trophic viruses that have pandemic level infectivity risks (31).

In summary, we demonstrate a mechanism of aging-associated skin infection risk. Age leads to circadian suppression in the skin, ultimately triggering a reduced antiviral barrier function in a BMAL1/CLOCK-dependent manner. Circadian pharmacological agents are able to rescue age related viral susceptibility in the skin, suggesting a therapeutic pathway for combating immunosenescence. Our findings have potentially wide implications for aging skin and may lead to new treatment strategies for prevention of cutaneous infection, wound care, and overall skin health in aging populations.

MATERIALS AND METHODS

In vivo wounding experiments

C57BL/6 wild type, Ifnar1−/−, B6.129-Arntltm1Bra/J (Bmal1−/−), ClockΔ19 mice (21) and LysM-Cre mice (32) were obtained from Jackson laboratories (Bar Harbor, ME). IL27p28fl/fl mice were gifts from Zhinan Yin (Biomedical Translational Institute, Jinan University) and Li Fan Lu (University of California San Diego). All mice were maintained under a specific pathogen-free environment. After anesthesia of the mice, 3-mm punch wounds were made on the back of each mouse at distinct times of day and collected 24 hours post-wounding. Tissue from non-wounded and wounded skin was dissected from each mouse, lysed in Trizol reagent (ThermoFisher, Waltham, MA), and kept at -80°C for RNA extraction, placed in OCT for immunofluorescence, or immunostained for flow cytometry. Information regarding RNA extraction, quantitative real-time PCR, immunofluorescence, or flow cytometry approaches can be found in Supplementary Methods. For aged/young skin studies, wounds were inflicted between 8:00 AM and 9:00 AM.
Young mice were between 3-6 months, and elderly mice were greater than a year. Male and female mice were used in this study.

**Keratinocyte cell culture**

Human primary keratinocytes were purchased from Thermo-Fisher Scientific. Cells were grown in a 37°C incubator in serum free Epi-Life cell culture medium (Gibco, Waltham, MA) supplemented with Epi-Life Defined Growth Supplement (EDGS) containing 0.05mM Ca^{2+}. NTERT 2G keratinocytes were a gift from the lab of Johann Gudjonsson of University of Michigan and cultured in Keratinocyte SFM media supplemented with EGF and BPE (Gibco) prior to use for luciferase and infection studies. Complete keratinocyte methods, including circadian synchronization, siRNA experiments, and infections can be found in supplementary methods.

**RNA-Seq gene expression and microarray gene expression data**

Publicly available non-human primate RNA-Seq gene expression data was downloaded from the Gene Expression Omnibus (GSE98965). The data was provided as a normalized expression matrix as calculated by Mure et al.(20). Publicly available microarray gene expression data from Geyfman et al.(19) was downloaded from the Gene Expression Omnibus(33) (GSE38625). Further computational analysis methods can be found in Supplementary Methods.

**Herpes simplex virus staining and quantitative PCR**

For a full description of viral infection, please see Supplementary Methods. Briefly, human or murine epidermis maintained in keratinocyte growth media and infected with 10,000 focus-forming units per sample of herpes simplex virus type 1 (HSV1) strain NS in the presence of vehicle, nobiletin or SR8728 (Sigma) at 5-10 µM. The epidermis was then either placed in 4% formaldehyde to fix overnight and subsequently stained for HSV1 antigen or lysed for DNA extraction and viral quantification.
**Statistical analysis**

All statistical tests were performed in GraphPad Prism. Throughout figures, box and whisker plots are shown as 10th to 90th percentile. Scatter plots with bar are shown as mean ± SEM. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001. Diagrams and visual abstract were created using BioRender software.

**Study Approval**

Animal procedures were performed in agreement with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocols were approved by Duke University’s Institutional Animal Care and Use Committee (Animal Welfare Assurance). Human tissue was used in accordance with Duke Institutional Review Board approval.

**Data Availability and Material Sharing:**

All data needed to evaluate our conclusions are present in the paper and/or the Supplementary Materials. Furthermore, all data points shown in graphs and values behind any reported means are provided in our Supporting Data Values file. All data and materials generated in this study will be made available upon request and completion of material transfer agreement per requirement by the original provider of cell lines and animal models. Murine skins (GSE38625) and baboon skins (GSE98965) datasets are publicly available (19, 20).

**Author Contributions:**

- Conceptualization: SK, VL, AM, DH, JZ
- Methodology: SK, VL, JS, AM, JZ, JC, DC
- Investigation: SK, VL, PK, MP, DC, AM, JZ, DW
- Visualization: SK, VL
- Funding acquisition: JZ, AM, JC
- Project administration: JZ, AM, JC, KD
- Supervision: JZ, AM, JC, KD
- Writing – review & editing: everyone

**ACKNOWLEDGEMENTS:**
This study was supported by funding from NIH 5R01AI139207 to ASM and JYZ, NIH 5R01AR073858 to JYZ, and NIH R01AI139425 to JC. We thank Helen Lazear and Drake Philip (University of North Carolina, at Chapel Hill) for providing Herpes Simplex Virus and insight and guidance on viral infections. We thank Drs. Russell Hall, Suephy Chen, Stacy Horner, and Andrew Alspaugh (Duke University, Durham) for comments, the lab of Johan Gudjonsson (University of Michigan, Ann Arbor) for NTERT 2G keratinocytes, and Zhinan Yin and Li Fan Lu (University of California, at San Diego) for providing Il27p28\[^{0/\beta}\] mice. We also thank Jacob Benton, Paula Marriottoni, Xin Ling, Margaret Coates, and Yingai Jin (Duke University, Durham) for their technical assistance and valuable comments.
REFERENCES


Figure 1: Aging skin exhibits diminished circadian, AVP, and IL-27 transcription

A

B

C

D

E

F

G

H

19
Figure 1: Aging skin exhibits diminished circadian, AVP, and IL-27 transcription. A) qPCR of *Bmal1, Clock, and Per2* in aged (n=14-16 1-year old) and young (n=32-34 1-month old) male murine skin. Graphs represent averages of relative mRNA ± SEM with GAPDH used for internal control. P-values were obtained via Student’s t-test. B) qPCR of *BMAL1, PER2* and *IFITM1* in human primary keratinocytes over serial passaging (n=2-3 donors per passage). Graphs represent averages of relative mRNA ± SEM with GAPDH used for internal control. C) qRT-PCR of *Oas1, Oas2, and Ifitm1* in aged and young murine back skins as described in (A). P-values were obtained via Student’s t-test. D) Immunostaining for OAS1a [orange], nuclei [blue] in aged and young nonwounded skin. Bar=25μm. E) qRT-PCR of AVP in young and old skin 24-hour post-wounding. NW=nonwounded skin, W=wounded skin. Graphs represent averages of relative mRNA ± SEM with GAPDH used for internal control. P-values were obtained via ANOVA with Multiple comparison. F) Immunostaining for CD301b [green], nuclei [blue] in aged and young unwounded skin. Bar=25μm. G) Flow cytometry showing reduced numbers of CD301b+ cells in aged skin compared to young skin (n=4 mice per group) as percent of total harvested live, single cells. P-values obtained via ANOVA with multiple comparisons. H) Histogram displays IL-27 production from CD11b+ (yellow) and CD301b+ (blue) cells compared to CD45- (red). Flow cytometry gating strategy included in Supplementary Figure S1.
Figure 2: Circadian rhythm transcriptional networks regulate antiviral genes in mammalian skin

A

Gene  
Arntl  
Oas1a

Expression

Time (h)

0 10 20 30 40 50

B

Gene  
ARNTL  
IFIT2

FPKM

Time (h)

0 5 10 15 20

C

Oas1a

Relative mRNA levels normalized to WT skin

WT  Bmal1–/–

D

OAS1a  OAS1a Nuclei

WT

Bmal1–/–

E

Oas1a

Relative mRNA levels normalized to WT skin

AM  PM

F

Oas1a

Relative mRNA levels normalized to NW AM skin

AM  PM

G

Oas1a

Relative mRNA levels normalized to NW skin

AM  PM

Iftim1

Relative mRNA levels normalized to WT skin

AM  PM

Iftim1

Relative mRNA levels normalized to NW AM skin

AM  PM

Iftim1

Relative mRNA levels normalized to NW skin

AM  PM
Figure 2: Circadian rhythm transcriptional networks include antiviral genes in mammalian skin. A-B) Line plots showing rhythmic expression of circadian factors and AVP genes in (A) murine skins (GSE38625) and (B) baboon skins (GSE98965). Heatmaps including additional genes can be found in Supplementary Figure S3. C) qRT-PCR of Oas1a and Oas2 in intact skin of Bmal1−/− mice and WT littermates. (n=3 mice/group, with technical triplicates/mouse). Graphs represent averages of relative mRNA ± SEM with GAPDH used for internal control. P-values were obtained via Student’s t-test. D) Immunostaining for OAS1a [purple] in WT and Bmal1−/− skin. Nuclei [blue]. Bar=25μm. E) qRT-PCR of Oas1a and Ifitm1 in intact belly skin of ClockΔ19 mice and BALB/C WT littermates harvested at 5AM or 5PM. P-values were obtained via Student’s t-test. F) qRT-PCR of Oas1 and Ifitm1 in skin wounds of C57BL/6 inflicted at times indicated and harvested 24 hours later (n=4 mice in each group). P-values were obtained via Student’s t-test. Ifitm1 p-value =0.0725. G) qRT-PCR of Oas1a and Ifitm1 in skin wounds of ClockΔ19 mice and BALB/C WT littermates inflicted at the indicated time and harvested 24 hours later (E, G: n=3 mice/group with technical triplicates, except WT PM group which used 2 mice). P-values were obtained via Student’s t-test.
Figure 3: Circadian rhythms of wound induced AVPs require cytokine signaling

A

B

C

D

E

F

G
Figure 3: Circadian rhythms of wound-induced antiviral proteins require cytokine signaling.  
A) Heatmap of microarray expression of Bmal1−/− compared to heterozygotes intact murine skin as measured at Zeitgeber time 22 (GSE38625). B) Immunostaining for CD301b [green] and nuclei [blue] in WT and Bmal1−/− intact skin. Bar=25μm. Skin samples are the same sections displayed in Figure 2C. C) Flow cytometry of CD301b+ cells expressing IL27 as percent of total harvested live single cells and mean fluorescence intensity (MFI) of Il27p28 in Bmal1−/− skin and WT skin (n=3-4 mice per group). P-values were obtained via Student’s t-test. D) Histogram displays IL27p28 expression in Bmal1−/− (red line) and WT mouse skin (blue line). Gating strategy is shown in Supplementary Figure S4. E) qRT-PCR of Ifitm1 in skin wounds of WT or LysM-Cre.IL27p28fl/fl (n=3 mice per group). P-values were obtained via Student’s t-test. F) qRT-PCR of Ifitm1 in skin wounds of Ifnar1−/− mice (n=3-4 mice). G) qRT-PCR of type I interferons in WT C57BL6 mice wounded at 8AM or 8PM (n=4 mice per time point) as in (E) above. Graphs represent averages of relative mRNA ± SEM with GAPDH used for internal control. P-values were obtained via Student’s t-test.
Figure 4: Keratinocyte autonomous circadian rhythm regulates antiviral activity

A

B

C

D

E

F
Figure 4: Keratinocyte autonomous circadian rhythm regulates antiviral activity. A) qRT-PCR of *BMAL1, OAS1, OAS2*, and *MX1* in human primary keratinocytes synchronized via growth factor starvation and harvested every 6 hours (representative of three independent experiments). Graphs represent relative mRNA ± SEM with GAPDH used for internal control and relative to that of 0-hour time-point. B) qRT-PCR of *BMAL1, CLOCK, OAS1*, and *IFITM1* in *NTERT* keratinocytes transfected with small interfering RNA either non-silencing control (*siCtrl*) or specific for CLOCK (*siCLOCK*) or BMAL1 (*siBMAL1*) (n=3 biological replicates). Graphs represent averages of relative mRNA ± SEM with GAPDH used for internal control. P-values were obtained via Student’s t-test. C) qPCR of HSV1 gene UL29 in cell culture conditioned media of primary keratinocytes transfected with either *siCtrl*, *siBMAL1*, or *siCLOCK* 24 hours prior to infection with HSV1 (MOI 0.01). Knockdown efficacy is shown by qRT-PCR. Graphs represent averages of either relative DNA or mRNA ± SEM with GAPDH used for internal control. P-values were obtained via Student’s t-test. Primary keratinocytes from donors were pooled, n=3. D) Immunofluorescence of HSV1 (MOI 0.01) in human *NTERT* keratinocytes transfected with non-silencing *siCtrl* or *siBMAL1*. Bar= 160um. E-F) Knockdown efficacy of *BMAL1* in human *NTERT* cells is shown by qRT-PCR. ImageJ (Fiji) quantification of relative viral immunofluorescence normalized to nuclear staining. Box and whisker plots represent averages of relative immunofluorescence ± SEM (n=3 samples per group in E, n>400 cells per condition in F). P-values obtained via Student’s t-test.
Figure 5: Pharmacological augmentation of circadian rhythm reduces herpes simplex virus burden in a Bmal1/Clock-dependent manner

A

BMAL1-Luciferase reporter (RLU)

ZT (hours)

Vehicle

SR8278

B

BMAL1 Luciferase Reporter (RLU)

Vehicle SR8278

C

Vehicle

SR8278

H3V1

H3V1 nuclei

D

Relative H3V1 fluorescence normalized to nuclei

Vehicle SR8278

E

H3V1 UL29 DNA abundance relative to R14

Vehicle SR8278

F

siCtrl

Vehicle

siCtrl

SR8278

siBmal1

SR8278

G

Relative H3V1 fluorescence normalized to nuclei

siCtrl

Vehicle siCtrl

SR8278 siBmal1

H

H3V1 UL29 DNA abundance

In conditioned media

siCtrl

siBmal1

siCLOCK

ns

****

****

ns
Figure 5: Pharmacological augmentation of circadian rhythm reduces HSV1 infection in a BMAL1/CLOCK-dependent manner. A-B) Bmal1-luciferase reporter assay. Human NTERT keratinocytes were transduced with Bmal1-luciferase reporter construct and growth supplement starved overnight before treatment with vehicle or 10 μM SR8278. Cells were harvested (A) every 6 hours and (B) at 24 hours for measurements of relative luminescence unit (RLU) (n=4 samples). P-values were obtained via Student’s t-test. C) Immunostaining for HSV1 antigen in human epidermal explants infected with HSV1 and treated with vehicle or 10 μM SR8278. Bar=500um. D) ImageJ (Fiji) quantification of viral immunofluorescence normalized to nuclear staining (n>5000 cells quantified per condition). P-values were obtained via Student’s t-test. E) qPCR of HSV1 viral gene UL29 relative to human KRT14 in epidermal skin infection (n=4 skin explants). P-values were obtained via Student’s t-test. F) Immunostaining for HSV1 antigen in human NTERT keratinocytes (MOI 0.01) transfected with siCtrl or siBMAL1 and supplemented with vehicle or 10 μM SR8278. Bar=160um. G) ImageJ (Fiji) quantification of relative viral immunofluorescence normalized to nuclear staining. Quantification of siCtrl (vehicle) is same data as used in Figure 4E (n>400 cells per condition quantified). P-values were obtained via Student’s t-test. H) qPCR of HSV1 viral gene UL29 in cell culture conditioned media of primary keratinocytes (MOI 0.01) transfected with siBMAL1 or siCLOCK and infected with HSV1 (n=3). Graphs represent averages of relative viral DNA or viral protein immunofluorescence normalized to human KRT14 or nuclear staining ± SEM. P-values obtained using ANOVA with multiple comparisons.
Figure 6: Antiviral immune decline of aging skin can be rescued by a circadian enhancer treatment

A

**Oas1a**

Relative mRNA expression to nonwounded wild type

Aged WT  | Aged Bmal1−/−
---|---
NW | W

**Oas2**

Aged WT  | Aged Bmal1−/−
---|---
NW | W

**Ifitm1**

Aged WT  | Aged Bmal1−/−
---|---
NW | W

B

HSV1 UL29 DNA abundance relative to Kit14

Young | Aged
---|---
Vehicle | SR8278

C

HSV1 UL29 DNA abundance expression relative to Kit14

D

HSV1 UL29 DNA abundance in conditioned media

E

HSV1 UL29 DNA abundance in conditioned media

F

A diagram showing interactions between various factors such as keratinocytes, BMAL1, CLOCK, and immune cells (e.g., T cells, CD301+). The diagram also includes cytokines and IFN pathways.
Figure 6: Antiviral immune decline of aging skin can be rescued by a circadian enhancer treatment. A) qRT-PCR of Oas1a, Oas2, and Ifitm1 in Bmal1+/− and wild type skin that was wounded and collected 24 hours later (n=3-6 mice per genotype). P value obtained with ANOVA with multiple comparisons. B) qPCR of HSV1 UL29 gene relative to murine Krt14 in HSV1 infected aging (>365 days) and young mouse (2-6 months) epidermis (n=3 skin explants per group). P-values were obtained via Student’s t-test. C) qPCR of viral UL29 relative to murine Krt14 in HSV1 infection of aging mouse (>365 days) epidermis treated with vehicle or 10uM SR8278 (n=3 skin explants per condition). P-values were obtained via Student’s t-test. D-E) qPCR of HSV1 UL29 in cell culture conditioned media of infected P2 and P8 keratinocytes treated with vehicle or 10uM SR8278 (MOI 0.01). Graphs represent averages of relative HSV1 UL29 DNA normalized to Krt14 ± SEM. P values obtained using Student’s T test (n=3 per group). F) Working model of aging-associated decline of cutaneous innate antiviral immunity. Circadian rhythm factors BMAL1 and CLOCK regulate expression of cutaneous antiviral proteins through both keratinocyte-autonomous and leukocyte-mediated processes. IL-27 conveys a time-of-day response and type I interferon signaling ensures a robust antiviral immunity. Aging-associated circadian decline decreases cutaneous antiviral immunity, while pharmacological means of circadian enhancement increases it.